FINAL REPORT

DEVELOPMENT OF VACCINES TO THE MYCOTOXIN T-2

ONR Contract No. N0014-83-C-0441

INTRODUCTION

This report presents the work carried out during the 21 months period from May 1983 to February 1985, on ONR Contract No. N0014-83-C-0441; Development of vaccines to mycotoxin T-2. T-2 is a trichothecene mycotoxin which is both acutely and chronically toxic. Its adverse effects range from skin necrosis to severe leukopenia and death, and the compound is also a potent immunotoxin. Currently there are no known prophylactic or therapeutic treatments for T-2 poisoning other than removal of the compound or prevention of exposure. This project is designed to explore the possibility that anti-idiotypic antibodies (anti-id-ab) to anti-T-2 antibodies can be used in an immunization protocol to prevent the adverse effects of T-2.

Development of Monoclonal Antibodies to T-2:

Being a small, non-immunogenic molecule, T-2 has to be presented as a hapten-carrier conjugate for immunization. Initially we proposed to couple several protein and carbohydrate carriers to T-2 toxin through its various chemical moieties including the epoxide group. However, the task of haptenization and immunization of T-2, was not carried out, as this work had already been carried out at USUHS by Drs. Hunter and Finkelman, and we were advised by our project officer, Dr. Majde, to collaborate with them. Initially we could obtain a small sample of polyclonal antibody containing serum from them, as monoclonals were not yet produced.

Development of an Assay System

Our proposed approach was to develop a radioimmunosassay system using [^{3}H]T-2. Experiments using [^{3}H]T-2 (a gift by Dr. Wannamaker, USAMARID) and polyclonal antibody (provided by Dr. Hunter) did not demonstrate any activity. Modifications of the assay system such as incubation time, temperature, use of anti-Fab alone as a second antibody or Cowan's Staph A as the precipitating

agent or using both anti-Fab and the Cowan's Staph A, did not precipitate any radiolabeled material.

As the polyclonal antibody supplied by Dr. Hunter was detected and titered by an enzyme-linked immunoassay ELISA, we decided to adopt and standardize the optimum conditions required for the ELISA. After initial screening with several commercially available brands of ELISA were determined. These include coating the plates with 25 µg/ml of T-2-BSA, blocking the unbound sites with 100 µg/ml of poly L-glutamine, and using an affinity purified anti-Fab (a generous gift from Dr. McClintock, NIDR, NIH). Subsequent characterizations of monoclonal and polyclonal sera were carried out using the above conditions.

Purification of Polyclonal and Monoclonal Antibody:

Simultaneously working with the standardization of the assay system to detect anti-T-2 antibody in the antiserum, the possibility of making an affinity column to purify the polyclonal antiserum to T-2, was also explored. At this point affinity purified anti-T-2 antibody was intended to use for the develoment of anti-idiotypic antisera.

In our earlier experiments to make hapten-carrier conjugates, we had focussed on the development of a T-2-hemisuccinate (T-2-HS), which can then be coupled through a carbodiimide reaction to amino groups on proteins and other molecules. Experiments were performed according to Chu et al., (Appl. Environ. Microbiol. 37: 104, 1979), where in T-2 toxin spiked with a small amount of [^{3}H]T-2 was dissolved in dimethylformamide and reacted with succinic anhydride in the presence of pyridine. The reaction was carried out in dark at 100° C in a paraffin oil bath with constant stirring for 4 hours. The chloroform extract obtained after decolorising and washing the reaction mixture, yielded 2% of T-2 toxin in T-2-HS form.

In order to overcome some of the draw backs encountered in this method such as high temperature, duration of reaction procedure, discoloration of the product and others, we developed a new rapid and simple method of T-2-HS formation. This reaction can be carried out at room temperature, in a chloroform phase, without heating. Under optimal conditions complete conversion of T-2 to T-2-HS can be achieved within 2 hours. The resultant product which is in

chloroform phase, on drying yields a colorless powder with a recovery rate of 66% as determined by spiking with [^{3}H]T-2 toxin.

The T-2-HS, prepared by the new method was conjugated to derivatised sepharose 4B beads (Sigma), at room temperature in the presence of water-soluble carbodiimide. Unreacted groups in the sepharose were blocked by an additional reaction with acetic acid and the gel was washed extensively. The washed gel was packed into a column and tested for its ability to bind and purify the polyclonal antibody provided by Dr. Hunter.

Using this column, subseqent batches of ascietes fluid containing monoclonal anti T-2 antibody provided by Dr. Hunter were purified, and titrated by ELISA decribed earlier.

Monoclonal Anti-T-2 Antibody:

Two mouse monoclonal antibodies (clone 12C12 and clone 15H6), that bind to the trichotheclene myclotoxin T-2 were produced by Dr. Hunter *et al.* (Appl. Environ. Microbiol. 49: 168, 1985). Both the clones produce antibodies belonging to the class IgG, with kappa-type light chains.

Production of Anti-idiotypic Antibody:

Three different immunization schedules were used to produce antidiotypic antisera in Balb/C mice:

1. Affinity purified monoclonal anti-T-2 antibody coupled to KLH (Sigma).
2. Affinity purified monoclonal anti-T-2 antibody coupled to affinity purified goat IgG (a gift from Dr. Finkelman).
3. Affinity purified monoclonal anti-T-2 antibody coupled to monoclonal anti mouse IgD (B6 anti-Balb/C IgD).

The immunization schedule 2 was adopted following the successful use by Drs. Hunter and Finkelman in the production of original anti-T-2 antibody. The hypothesis for this scheme is, when the conjugate is given along with purified goat anti-IgD, carrier specific helper - T Cells for goat IgG (which is conjugated monoclonal anti-T-2 antibody) are stimulated. Since anti-T-2 antibody will be a poor antigen being an isologous immunogen, it was reasoned that this immunization schedule can elect an anti-idiotypic response. The Schedule 3 was visualized with a hope that it will also recruit T-cell help for idiotypic determinants.

The three conjugates mentioned above were prepared using the described by Bona et al. (1976). Briefly, equal parts of purified antibody and KLH or goat IgG, or B6 anti-Balb IgD were mixed (0.5 mg/ml) in the presence of 0.05% glutaraldehyde. The reaction was carried until the mixture became opalescent, and then stopped by adding lysine to a final concentration of 0.05%. The resultant conjugate was extensively dialyzed. The immunization schedule followed, was as described by Gheuens et al. (1981). Balb/C mice (8-10 weeks) were injected with the conjugates emulsified in Freund's complete adjuvant. In the case of goat-Ig coupled to the anti-T-2, 50 mg of goat anti-IgD was given simultaneously. Each mouse was injected with 100 µl of emulsion distributed among the hind foot pads, inguinal and axillary regions. Five days later a similar dose of antigenic preparation in Freund's incomplete adjuvant was given. The mice were boosted weekly with the respective conjugates in saline. The sera from these mice were examined for the presence of anti-idiotypic antibody, after 4th injection onwards.

The anti-idiotypic antibody was detected by ELISA, using the monoclonal anti-T-2 antibody coated 96 well plates and screening for a positive reaction and then for inhibition of that reaction by free T-2 toxin. Initially the serum samples were screened at a dilution of 1:100, using a fixed concentration of free T-2 toxin such as 25 µg/ml for inhibition. The difference in OD units between the wells unblocked with T-2 and those blocked with free T-2 toxin was taken as the OD value demonstrating the anti-idiotypic antibody. The positive Id reaction was further confirmed using 96- wells plates coated with monoclonal antibody to paroxin (which is also IgG1), a gift by Dr. Hunter. Anti-idiotypic antibody was detected as early as after 4 injections. The titer of the antibody levels rose in accordance with the subsequent booster injections. However, no further increase was observed after 7 injections.

Of the three immunization schedules followed, mice receiving Schedule 3 and 1 demonstrated comparatively higher levels of anti-Id-antibody as early as after 4 injections, than those receiving Schedule 2, as determined by ELISA. Mice receiving anti-T-2 antibody coupled to B_6 anti Balb IgD (Schedule 3) have demonstrated a titer of 1:2000 to 1:4000 after 5th and 6th injection, which did not increase thereafter. Mice immunized with Schedule 1 had a titer of

1:1000 to 1:4000 and those with Schedule 2 had 1:200. Further boosting of the group with Schedule 2 did not increase the levels of antibody.

The Schedule 1 and 3 were used subsequently to immunize additional number of mice to expand the stock of mice producing anti-Id-antibody. The antibody levels detected in mice receiving various conjugates (Syngenic immunization) are summarized in Table 1.

Expansion of Immune Mice Producing Anti-Id-antibody to Mycotoxin T-2:

Syngeneic Immunization in Balb/C Mice

Two different immunization schedules described below were employed to develop anti-Id-antibody containing sera in Balb/C mice. Since the monoclonal anti-T-2 antibody antigen used for immunization is also of the Balb/C origin, the antigenic region (at the antibody combining site) on the immunoglobulin molecule will be too small and may act as a hapten. Hence, the monclonal anti-T-2 antibody (obtained from Dr. Hunter) was coupled to various proteinaceous carrier molecules.

1. Affinity purified monoclonal anti-T-2-antibody (clone 12C12) coupled to Keyhole limpet Haemocyanin (KLH).
2. Affinity purified moncolonal anti-T-2-antibody coupled to monoclonal anti-mouse IgD (clone 10-4-22).

Three batches (30 animals each) of Balb/C mice were immunized according to the above schedule. The serum from immune mice were titrated after the 4th, 5th, and 6th injections by inhibition of ELISA. The antibody level detected in mice receiving various conjugates are summarized in Table 1. Appreciable amounts of anti-idiotypic antibodies to T-2 toxin were produced in a syngeneic system by both the conjugates. However, booster doses did not facilitate an increase in the antibody levels. On the contrary a marked decrease in the antigen-specific antibody levels were observed. This may be due to the production of regulatory idiotypes.

The previous studies by Liberman (PNAS, 68:2008, 1971) have shown that immune responses to Balb/C mice derived IgA myeloma proteins in various strains of mice is dependent upon differences in the immunoglobulin allotype as well as the H-2 type of the recipient strains. In view of these

observations, the production of anti-idiotypic antibodies to T-2 toxin was extended to the mice belonging to the SJL/J ($H\text{-}2^d$ haplotype, IgG of 'b' allotype) and A/J ($H\text{-}2^a$ haplotype with IgG of 'd' allotype) strains.

The following immunization schedules are employed:

1. Monoclonal anti-T-2 antibody (clone 12C12) coupled to goat anti-mouse IgG.
2. Monclonal anti-T-2 antibody (clone 12C12) coupled to KLH.

Mice were test bled after the 4th, 5th, 6th, and 7th immunizations for evaluation of antibody levels. Presence of anti-idiotypic antibody was determined by inhibition of ELISA. A brief summary of these immunizations is given in Table 2. These mice are producing higher levels of serum anti-idiotypic antibody. However, antigen specific antibody could be detected by 50-60% (as inhibited by the free toxin). The remaining high background activity (0.45-0.5 OD units) which was not blocked by free toxin may be accountable for the other types of anti-idiotype antibodies directed against other regions of the immunoglobulin molecule, as it is an allogeneic system.

Standardization of Leucopenia in Mice:

To examine the efficacy of the anti-idiotypic antibody as a prophylactic agent, *in vivo* assays need to be established. Initially a single I.P. injection of varying concentrations of T-2 toxin was used in the mice and their peripheral white cell counts were monitored for up to 4 weeks. As the results obtained by this route of dosing were not consistent, subsequently the oral route was investigated. The mice were orally dosed with T-2 toxin in the concentration of 1 and 2 µg per gm body weight, and their white cell counts were monitored for various time periods up to a week (Fig. 1). Increase in blood white cells were observed in both dose levels up to 96 hours and decreased thereafter. There was an initial drop in cell count after 3 hours of administration in both the cases. Drop in cell count persisted until 6 hours in case of 2 µg/gm group, and increased gradually until 96 hours and decreased later. The effect was less dramatic at 1 µg/gm level.

The rise in the WBC count in the peripheral blood is possibly due to the recruitment of lymphocytes from regional nodes spleen and other lymphatic organs. The mice orally dosed with 2 µg/gm, were also examined for, the total

cell count in thymus and spleen at various time points (Fig. 2). There was a slight increase in cell population in the spleen at 1 hour after administration of the toxin, followed by a drop at 3 hours, which then increased until 24 hours, dropped again at 48 hours. At this point, WBC in the peripheral blood was high suggesting the migration of cells from lymphatics to the circulation. At 72 hours, there was a slight raise which decreased subsequently. A slightly different but similar pattern was observed in thymus cell counts. Initial drop at 1 hour was followed by an increase up to 6 hours, which decreased until 48 hours, slightly elevated at 72 and 96 hours, decreased thereafter.

Protective Role of Immunization on T-2 Toxin Exposure:

Development of immunity to T-2 toxin exposure, was the primary goal of this project. During the second half of the contract period animal experiments were conducted to examine several important questions relevant to protective immunity to T-2 toxin.

It was determined whether active immunization with T-2 toxin (T-2-HS coupled to KLH and monoclonal anti-IgD) would confer protection upon subsequent exposure to T-2. Conjugates such as T-2-HS coupled to KLH and anti-IgD were prepared and used to immunize rats and mice. The immune mice and rats producing appreciable levels of anti-T-2 antibody were challenged orally with free T-2 toxin (1 and 2 µg/gm body weight) by gavage. Non-immune mice and rats served as controls. Leucopenia was monitored periodically at different time intervals. No dramatic difference (Fig. 3) in the onset of leucopenia and its reversal to normal levels was observed in immune animals. These experiments need to be repeated using higher levels of T-2 toxin challenge.

We also evaluated whether active immunization to T-2 would confer protection to dermal challenge. CAM rats immunized with T-2-HS-KLH conjugates (with and without boosting) and controls were shaved on their back and painted with various levels of T-2 toxin including 0.0, 0.05, 0.1, and 0.2 micrograms in a 50% ethanol solution. The animals were observed for various time periods for the development of scirpinoid reaction characteristic of T-2 toxin. The data is summarized in Tabel 3. The immune animals developed delayed and less intense reactions which were of shorter duration. The typical reaction as

characterized by a red scirpinoid ring was visible at 16 hours in non-immune controls. These reactions persisted for 3-4 days followed by alopecia. These data suggest the protective role of active immunization.

We also determined whether anti-T-2 antibody can offer protection upon passive transfer. If so, anti-T-2 antibody could serve as an effective antidote for dermal exposure to T-2. This point was examined by two series of experiments as decribed below:

1. Neutralization of T-2 Toxin by Anti-T-2 Antibodies in Vitro

The various concentrations of monoclonal anti-T-2 antibodies (clone 15H6 and 12C12) were incubated with T-2 toxin at 37° C for two hours. The mixture was then smeared onto the shaved skin of the rats. The rats were examined for the development of skin necrotization reactions (Table 4). Both polyclonal and monoclonal antibodies demonstrated the ability to neutralize the toxicity of T-2 toxin as evident by minimal or no reactions developing with the toxin-antibody incubation mixture.

2. Passive Protection by Anti-T-2 Antibody Upon Exposure to T-2 Toxin

Non-immune rats were injected intradermally with varying concentrations of monoclonal anti-T-2 antibody and unfractionated rat anti-T-2 serum. Four to 6 hours later the injected area was smeared with lug of T-2 toxin in an alcohol solution. The development and intensity of the scirpinoid reaction was observed and recorded. Immune rats and saline injected rats served as positive and negative controls. The results from this experiment suggest the passive protection offered by anti-T-2 antibody. Both polycolonal and monoclonal antibody were able to neutralize the toxin on the skin surface, thereby inhibiting necrotization.

TABLE 1. Production of Anti-idiotypic Antibody in Balb/C Mice

A. Immunization with Monolconal Anti-T-2 Antibody Coupled tc KLH.

# of Injections	Titer (Antiserum Dilution)*
4	1:400 to 600
5	1:700 to 1000
6	1:2000 to 4000
7	1:2000 to 4000
Booster (After resting for 45 days)	1:1000 to 4000
Booster II	1:500 to 1000
Booster III	1:400 to 1000

B. Immunization with Monoclonal Anti-1-2 ab Coupled to Monoclonal Anti-IgD (clone 10-4-2, Bb anti-Balb/C Igd).

# of Injections	Titer (Antiserumdilutions)*
4	1:500 to 900
5	1:1000 to 2000
6	1:3000 to 5000
7	1:2000 to 4000
Booster I	1:800 to 1000
Booster II	1:400 to 700
Booster III	1:300 to 400

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* Titer values represent the highest dilution of antiserum where complete activity is blocked by 25 µg/ml of free toxin by ELISA.

TABLE 2. Production of Anti-Idiotypic Antibody in SJL/J and A/J Mice

Conjugate Injections Anti-T-2 Antibody Coupled to Goat Anti-Mouse IgG	Antibody Titer †
4	1:200 to 400
5	1:500 to 1000
6	1:2000 to 4000
7	1:2000 to 4000
Booster I	1:2000 to 4000
Booster II	1:2000 to 3000

Anti-T-2 Antibody Coupled to KLH	Antibody Titer †
4	1:200 to 400
5	1:600 to 950
6	1:1000 to 4000
7	1:2000 to 7000
Booster I	1:5000 to 8000
Booster II	1:5000 to 8000

† The highest dilution of antiserum where idiotypic antibody could be detected. Binding of anti-idiotypic antibody to monoclonal anti-T-2 antibody (Clone 12C12) coated plates was inhibited by free toxin (50-60% inhibition).

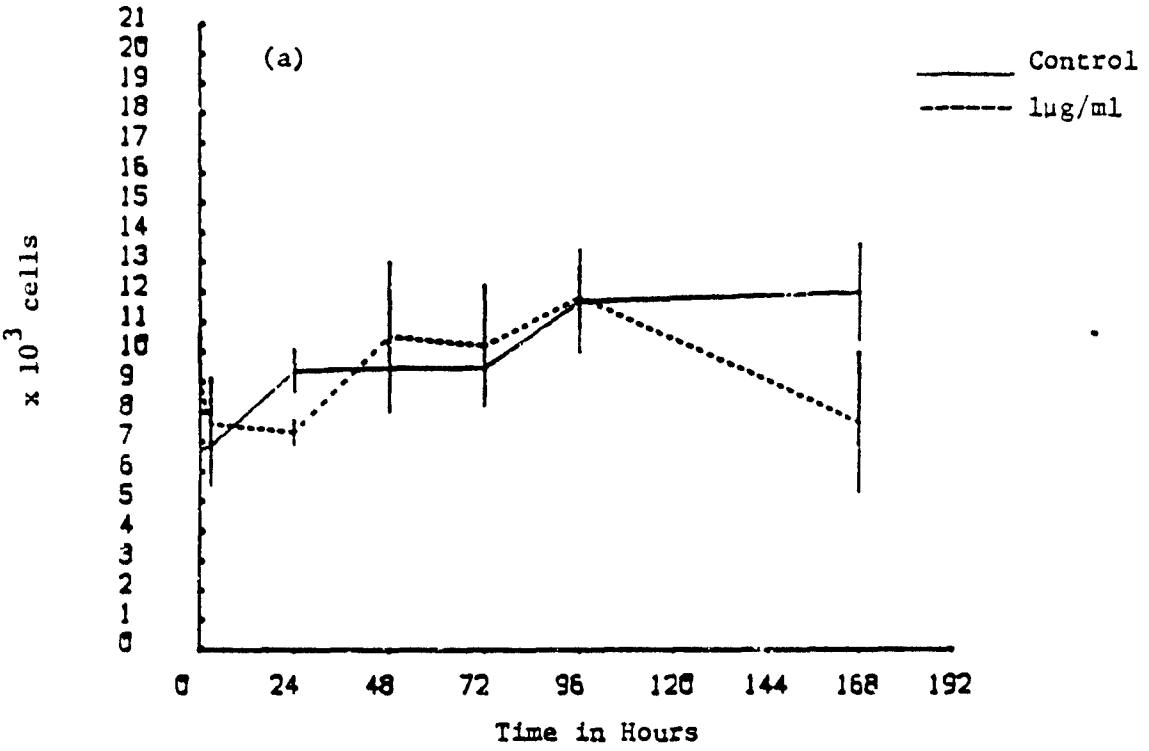
TABLE 3. Dermal Protection Following Active Immunization

Dose of T-2 Toxin (ugs)	Skin Reactions [2, 3]				
	Hours				
Immune Rats [1] (T-2-HS-KLH Conjugate)	16	24	48	72	96
0.05	±	+	+	±	-
0.1	±	+	+	±	-
2.0	+	++	++	+	±
Controls[1]					
0.05	++	++	+++	+++	++
0.1	++	+++	+++	+++	++
2.0	+++	++++	++++	+++	+++

[1] 6 animals in each group.
[2] Skin reactions were observed up to 7 days after exposure to T-2.
[3] Skin necrotizations are graded as follows:

± = Appearance of slight inflammation
\+ = Inflammation positive.
++ = A circular ring-like reaction
+++ = A circular ring with defined margin, increased inflammation throughout the area
++++ = A circular ring with definite margin, with intense inflammation

Fig. 1. Induction of Leucopenia in BALB/C mice by T2-toxin (peripheral blood



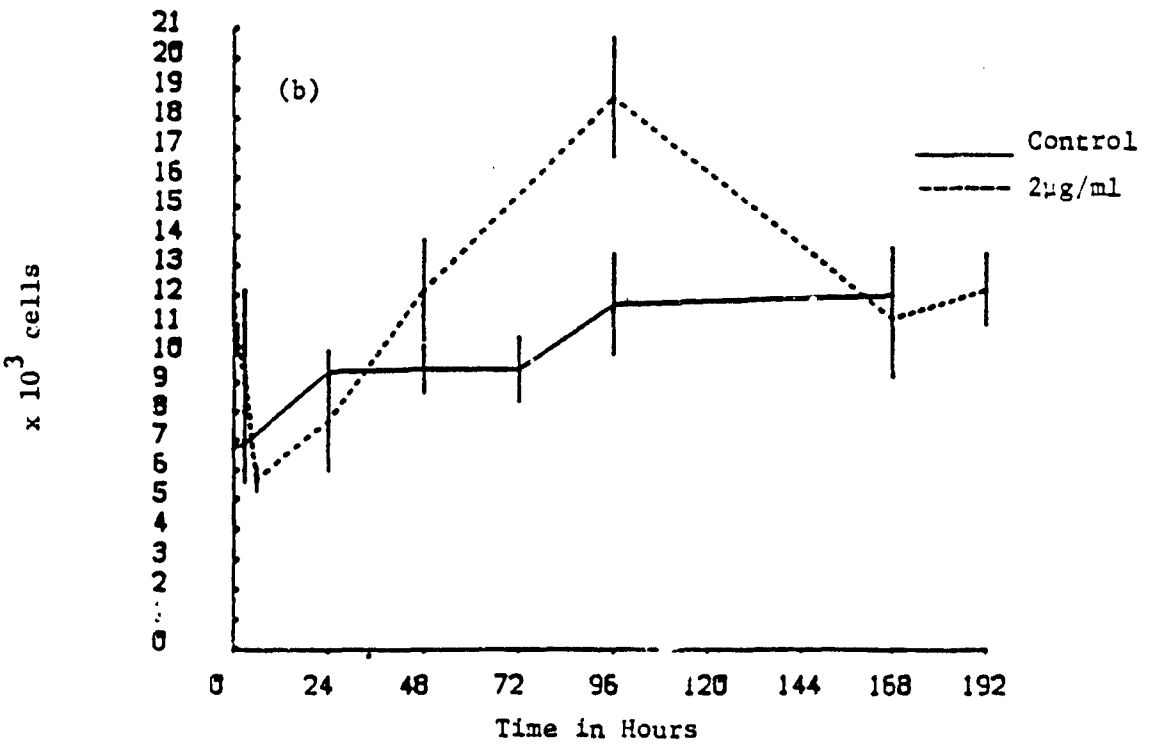


Fig. 2. Induction of Leucopenia in BALM/C mice
Effect of T2 toxin on thymus and spleen

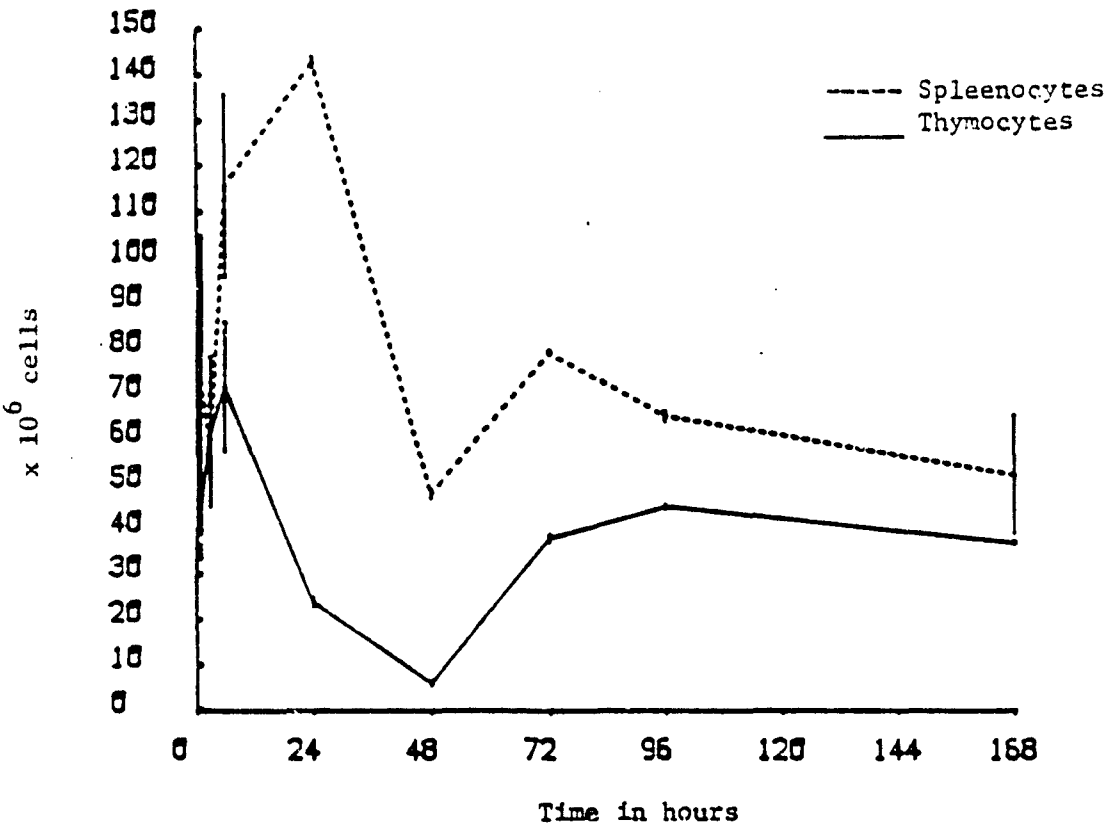
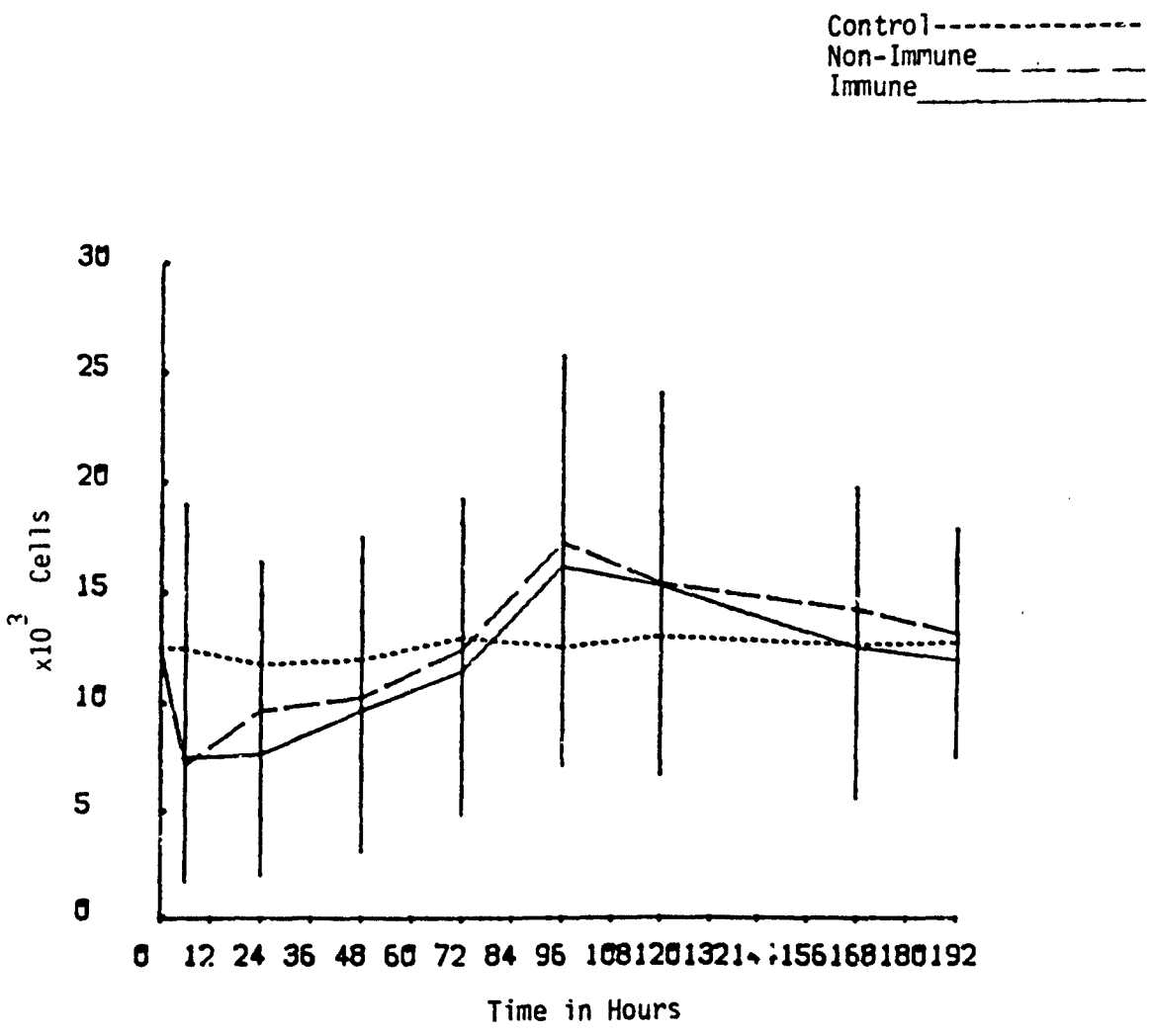


Fig. 3 Protective Role of Immunization on T-2 Toxin Exposure
(in vivo)



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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

An ELISA System to detect and titer antibody to Mycotoxin T-2 was standardized. A new rapid simple method of making T-2 hemisuccinate (T-2-HS) was developed. The merit of the new method over the existing one accounts for its simple, less time-consuming procedure, which can be carried out at room temperature, with as high a yield of 66%. T-2-HS so developed was conjugated to derivatised sepharope-4B beads to make a column to affinity purify the antibody to Mycotoxin T-2. Polyclonal antisera to T-2 toxin was produced by immunizing rats

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20. ABSTRACT (Continued):
and mice with T-2-HS coupled to KLH, goat antimouse IgG and monoclonal anti-mouse IgD.

Affinity purified antibody to T-2 was employed in immunizing mice for the development of anti-idiotypic antibodies. Initially syngeneic immunization were carried out in Balb/c mice. Although anti-idiotypic antibodies were produced initially, they were suppressed very shortly by the development of regulatory idiotypes. Allogeneic immunization using B 1b/c derived monoclonal Anti-T-2 coupled to KLH and goat IgG was carried out in SJL/J and A/J mice. A high level of antibodies (1:2000 to 5000 dilutions of antisera) were detected, out of which only 50-60% of the binding was found to be inhibited by free toxin.

Protective role anti-T-2 antibodies upon passive transfer, was examined. Both monoclonal and polyclonal antibodies to T-2, were found to neutralize T-2 toxin in vitro. Upon passive transfer to skin intradermally, inhibition of skin necrotization effect due to T-2 toxin was demonstrated, suggesting, role of an effective antidote. Protective immunity to in vivo exposure to T-2 toxin, upon active immunization with T-2-HS was also examined. Skin necrotization effect of T-2 toxin was completely inhibited in immune rats. However, the in vivo effect of Oral T-2 toxin, as measured by leucopenia in actively immunized rats, was not dramatically significant.